Enzyme mediated concise synthesis of NH-Fmoc-S-Trityl-C^α-Methyl Cysteine

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Abstract: A concise, chiral auxiliary free, methodology for the preparation of orthogonally protected C^α-Methyl cysteine has been developed. Curtius rearrangement of malonate half ester from PLE hydrolysis^{1, 2} and simultaneous Fmoc protection using Titanium (IV) Isopropoxide yielded Fmoc protected amino ester that is then transformed into (*R*) NH-Fmoc-S-Trityl-C^α-Methyl Cysteine in three steps. For the synthesis of the *S* enantiomer of the protected amino acid, enantiomerically enriched CO₂-*t*-Bu half ester derived from PLE hydrolysis was subjected to Curtius reaction followed by Fmoc protection using Titanium (IV) Isopropoxide which is then converted to (*S*)-NH-Fmoc-S-Trityl-C^α-Methyl Cysteine in two steps.

Keywords:

C^α-methyl Cysteine; Porcine Liver Esterase; Enantioselective Synthesis; Orthogonal protection/deprotection.

Introduction: There has been increasing interest in orthogonally protected amino acid analogues in the field of peptide chemistry because of the convenient removal of protecting groups during solid phase peptide synthesis. Our primary interest in the synthesis of protected α -methyl substituted cysteine analogues with NH-Fmoc and S-Trityl protecting groups (Compounds **I** & **II**) is because these derivatives are known to be widely used as key scaffolds in the synthesis of several biologically important molecules. Unnatural cysteine analogues with a C^α-methyl group are known to be a key component in several natural products like Largazole and derivatives.^{3, 4} The C^α-methyl group in an amino acid restricts the conformational mobility of the peptide backbone in a polypeptide resulting in improved resistance to chemical and enzymatic degradations.⁵⁻⁹ Additionally, cysteine and its analogues, when incorporated into polypeptides, can form disulfide bridges causing the peptides to fold.¹⁰⁻¹² Recently, C^α-methyl cysteine has been utilized in protein ligation for their ability to form in situ thioesters.¹³ There are several existent strategies for the preparation of C^α-methyl cysteine in its protected form.^{2, 14-19} Kedrowski reported the synthesis of both enantiomers of NH-Moz and S-*t*-butyl protected C^α-methyl cysteine² and later our group reported an improved method for the preparation of protected cysteine that was used in the synthesis of a glutathione analogue.²⁰ Both procedures utilize Pig Liver Esterase for the formation of the quaternary chiral center in an enantioselective fashion. Singh et al reported a procedure for the synthesis of NH-Fmoc-S-Trityl protected C^α-methyl cysteine.¹⁵ This method involves the use of a chiral auxiliary, *S* or *R*-Camphorsultam, in a stereoselective alkylation procedure to create the chiral quaternary center followed

by installation of the appropriate protecting groups. Our strategy is to utilize a chemoenzymatic approach producing protected C^α-methyl cysteine suitable for solid phase synthesis of peptidomimetics.

Experimental Results:

Initially we planned to develop a synthetic route enabling the preparation of the target cysteine analogues in a straightforward manner (Scheme 1). Triphenylmethanethiol (**1**) was reacted with sodium hydride in anhydrous THF to generate the thiolate anion. Addition of anhydrous CH₂Cl₂ caused the displacement of a single chloride to form alkylating agent **2**, which was then reacted with dimethylmalonate to provide compound **3**. Compound **3** was subjected to PLE hydrolysis in phosphate buffer at pH 7.4. However, PLE did not show activity towards substrate **3**. We speculate that the trityl group in substrate **3** is simply too large to allow the substrate to fit in the PLE active site.²¹ After the unsuccessful reaction as shown in Scheme 1, we had to follow the standard methodology reported by our group^{1, 20} to obtain the enantioenriched chiral half-ester needed for the preparation of both enantiomers of C^α-methyl cysteine.

Scheme 1: Attempted synthesis of chiral half-ester (**4**) by asymmetric enzymatic hydrolysis (PLE)

(*R*) NH-Fmoc-S-Trityl-C^α-Methyl Cysteine-OH (**I**):

For the enantioselective synthesis of the title compounds we adopted the procedure for the desymmetrization of malonate diesters.^{2, 20} The procedure previously utilized by our group provides the Moz protected amine. A novel approach to orthogonally protected NH-Fmoc-S-Trityl-C^α-Methyl Cysteine was developed to facilitate the incorporation of the amino acid into a poly peptide which is currently under investigation. The synthesis of the *R* enantiomer of NH-Fmoc-S-Trityl-C^α-Methyl Cysteine was accomplished as illustrated in Scheme 2. The malonate half-ester (**6**) was prepared by PLE hydrolysis in 94% ee. The half-ester (**6**) was treated with DPPA and triethylamine to provide the isocyanate intermediate. The crude isocyanate was quickly isolated and taken to the next step without further purification. The isocyanate was reacted with 9-Fluorenyl methanol in the presence of Titanium (IV) Isopropoxide to facilitate formation of the Fmoc protected amine in one step.^{22, 23} The resulting product (**7**) was then hydrolyzed with HCl to provide compound **8** in quantitative yield. Interestingly, the *S*-*t*-butyl and Fmoc groups remained intact under these conditions. There are reports suggesting that extended refluxing under acidic conditions can remove the *S*-*t*-butyl protecting group, but in our hands this was not observed.^{14, 17} We utilized Mercury (II) to readily remove the *S*-*t*-butyl group within 5 hrs. This reaction involves treatment of **8** with a mercuric acetate / TFA / anisole cocktail at room temperature. The resulting mercury species were washed with cold ether to remove soluble organic material and provide a gray precipitate. The precipitate was taken up in methanol and H₂S gas was bubbled through the solution for 30 minutes to reduce the mercurial species and liberate the free thiol. The resulting mixture was centrifuged to precipitate the fine particulate of mercury sulfide. The solution was decanted and passed through a 2.0μ HPLC syringe filter to remove trace particulate. The free thiol was immediately taken to the next step without further purification. In the final step, (*R*)-NH-Fmoc-C^α-Methyl Cysteine-OH was reacted with Triphenyl methanol in the presence of TFA to provide (*R*)-NH-Fmoc-S-Trityl-C^α-Methyl Cysteine-OH (**I**) in 59% yield over two steps. The final product was purified by reverse phase HPLC and chiral HPLC was performed on the purified product and the %ee was

determined to be 94%. Product **I** was further purified by recrystallization from acetonitrile/water (73% recovery) and the %ee of the crystalline product was improved to >99% (determined by chiral HPLC).

Scheme 2: Synthesis of (R) NH-Fmoc-S-Trityl-C^α-Methyl Cysteine-OH (**I**) from malonate diester (**5**)

(S) NH-Fmoc-S-Trityl-C^α-Methyl Cysteine-OH (**II**):

Compound **9** was prepared in 90% overall yield according to the reported procedure from **6**.² The half ester **9** was reacted with DPPA and triethylamine to provide an isocyanate, which was worked up and taken to next step without purification. In the next step the crude isocyanate was reacted with 9-Fluorenylmethanol in presence of Titanium (IV) Isopropoxide to facilitate the formation of NH-Fmoc protected amino ester **10** in 51% yield over two steps. Treatment of compound **10** with the Mercury (II) acetate/anisole/ TFA cocktail followed by H₂S gas cleaved both S-*tert*-butyl and CO₂-*tert*-butyl groups to provide the NH-Fmoc protected C^α-methyl cysteine amino acid in quantitative yield. In the final step, (S)-NH-Fmoc-C^α-Methyl Cysteine-OH was reacted with triphenylmethanol in TFA to provide (S) NH-Fmoc-S-Trityl-C^α-Methyl Cysteine-OH (**II**) in 82% yield. The final product was subjected to HPLC (chiral stationary phase) analysis and the %ee was determined to be 92%. The retention times of both *R* and *S* enantiomers were compared and the peaks corresponding to products were identified by MS analysis of the collected fractions from HPLC using chiral stationary phase. Amino acid, **II**, was further recrystallized from acetonitrile / water (84% recovery) in >99% ee as determined by chiral HPLC.

Scheme 3: Synthesis of (S) NH-Fmoc-S-Trityl-C^α-Methyl Cysteine-OH (**II**) from half ester (**9**)

Conclusion:

The designed synthetic approach to the target compounds **I** & **II** as shown in Scheme 1 was not successful as the Pig Liver Esterase enzyme did not show activity towards the malonate diester, **3**. We

were able to develop a short and concise synthetic methodology to prepare both enantiomers of NH-Fmoc and S-Trityl protected C^α-Methyl Cysteine-OH in high optical purity (>99% ee) from PLE hydrolysis and further recrystallization in the final step. The optical purity of the amino acids were determined by chiral HPLC and the absolute stereochemical configurations were assigned by comparing the specific rotation values to the literature values.¹⁵

Acknowledgements:

We are thankful to Dr. Julie A. Pigza for the valuable discussions regarding 2D-NMR data. We thank the National Science Foundation for a CAREER Award (MCB-0844478). We thank the National Science Foundation for the Mass Spectrometer and NMR Spectrometer instrumentation grants (CHE-0639208, DBI-0619455, and CHE-0840390). We thank the American Chemical Society, the Mississippi Section of the American Chemical Society, and the Vice President for Research (Dr. Gordon Cannon) for Project SEED funding (SOT). We thank the Department of Chemistry and Biochemistry for continued support of our research programs.

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Graphic Abstract: